Design of Supported Membranes Tethered via Metal-Affinity Ligand-Receptor Pairs

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ABSTRACT Model lipid layers are very promising in investigating the complex network of recognition, transport and signaling processes at membranes. We have developed a novel and generic approach to create supported lipid membranes tethered by metal-affinity binding. By self-assembly we have generated various interfaces that display histidine sequences (6xHis) via polymer spacers. These histidine-functionalized interfaces are designed to allow specific docking and fusion of vesicles containing metal-chelating lipids. By means of surface plasmon resonance and atomic force microscopy we analyzed the formation and subsequently the structure of these solid-supported membranes. Although the affinity constant of single ligand-receptor pairs is only in the micromolar range, very stable immobilization of these membranes was observed. This behavior can be explained by multivalent interactions resembling many features of cell adhesion. The process is highly specific, because vesicle docking and bilayer formation are strictly dependent on the presence of metal-affinity ligand-receptor pairs. The surface accessibility and geometry of these tethered membranes were probed by binding of histidine-tagged polypeptides. The supported membranes show adsorption kinetics and values similar to planar supported monolayers. Using various combinations of metal-chelating and histidine-tagged lipids or thiols these metal-affinity-tethered membranes should make a great impact on probing and eventually understanding the dynamic dialog of reconstituted membrane proteins.

INTRODUCTION

In recent years solid-supported membranes have attracted much interest as they mimic biological recognition processes at cellular membranes (Sackmann, 1996; Tampé et al., 1996). Nonbiocompatible materials can be functionalized for biological purposes to suppress nonspecific binding or to mediate the transition between solid and soft matter linking life and material science (Nuzzo and Allara, 1983; Ulman, 1991; Prime and Whitesides, 1991). In addition, solid-supported lipid layers are an important prerequisite for the functional immobilization of membrane proteins (Tamm and McConnell, 1985). The biophysical properties of these interfaces can be investigated in detail by surface-sensitive techniques such as surface plasmon resonance (SPR), atomic force microscopy (AFM), ellipsometry, or reflection interferometry.

The classical way of forming supported membranes is to transfer the lipid layer by Langmuir-Blodgett or Langmuir-Schaefer techniques onto various substrates. Today the most common method is fusion of vesicles to a support (Tamm and McConnell, 1985), which can be bare substrates, such as gold or glass, or alternatively surfaces coated with polymers or self-assembled monolayers (SAMs). In the later case alkylthiol on gold or alkylsilanes on glass are commonly used (Kalb et al., 1992; Nollert et al., 1995). By lipid transfer on ultrathin hydrated polymers polymer-supported lipid layers have been created (Spinke et al., 1992; Kühner et al., 1994). Recently, supported lipid bilayers have been described where the membrane is anchored to the surface via flexible spacers (Cornell et al., 1997; Williams et al., 1997; Bunjes et al., 1997; Heyse et al., 1998; Bieri et al., 1999).

Chelator lipids have been developed into a very useful concept for immobilization, orientation, and two-dimensional organization of proteins at interfaces (Schmitt et al., 1994; Shnek et al., 1994). Metal-chelating lipids combine the unique properties of lipids and metal-chelating moieties for specific immobilization of histidine-tagged proteins. In these fusion proteins a short stretch of typically six histidines (6xHis-tag) is co-expressed with the protein of interest with minimal perturbation of its structure or function. This strategy ensures a unique orientation of the immobilized histidine-tagged fusion protein at well-defined interfaces. A complex is formed between the histidine tag and metal ions, mostly nickel ions, which are trapped forming a complex with the *N*-nitrilotriacetic acid (NTA) lipid headgroup.

SPR is widely used to detect molecular interactions of a ligand with an immobilized receptor (Rich and Myszka, 2000). The observed change in the SPR signal reflects a change of the refractive index or layer thickness at the sensor surface. The technique allows label-free real-time measurements. It was used to study, e.g., protein adsorption to SAMs (Sigal et al., 1996; Bamdad, 1998; Dorn et al., 1999), or ligand-receptor interaction (Kelly and O'Connell, 1993; Dorn et al., 1998b). From the measurements thermo-

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dynamic and kinetic information can be derived (O'Shannessy et al., 1993).

Here we compared three different architectures of solid-supported membranes. In the first approach (Fig. 1 *a*), a well defined planar lipid monolayer on hydrophobic SAMs of alkylthiols is formed (Plant et al., 1995; Dorn et al., 1998b, 1999). This system serves as reference in the characterization of tethered lipid membranes. To create supported membranes, which are tethered via ligand-receptor pairs, we build up (6xHis)-functionalized interfaces by self-assembly of histidine-tagged thiols or lipids. In one system, the self-assembled films are composed of histidine-tagged thiols that serve as a specific anchor for the immobilization of metal-chelating lipid bilayers (Fig. 1 *b*). In the second system, the (6xHis)-functionalized interface is generated by self-assembly of a lipid monolayer composed of histidine-tagged lipids (Fig. 1 *c*). Although rather similar in architecture, these two histidine-functionalized interfaces differ with respect to the lateral mobility of the histidine-tagged thiol or lipid.

These concepts appear to be a promising extension of the NTA-histidine system, which is largely used in biochemistry and molecular biology. In this study, metal-chelating lipids and histidine-tagged SAMs are used for specific vesicle docking and formation of ligand-receptor anchored membranes. These novel, so-called metal-affinity-tethered (MAT) membranes are stable and functional regarding specific binding of (6xHis) fusion proteins. The ligand-recep-

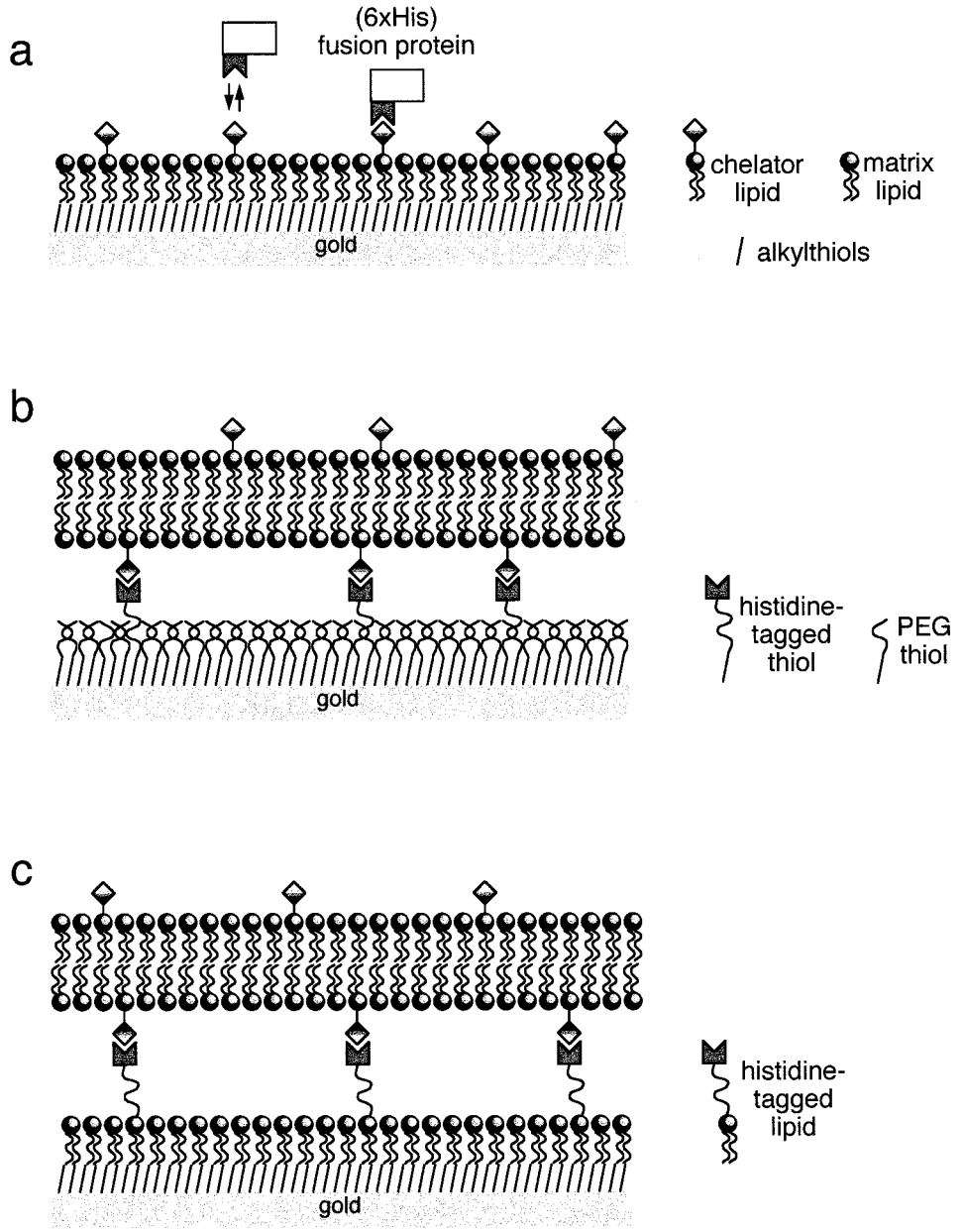

FIGURE 1 Different approaches to generate supported membranes. (*a*) Chelator lipid monolayer supported on a hydrophobic self-assembled monolayer (SAM) of alkylthiols. These planar lipid monolayers serve as the reference system in binding studies of (6xHis) fusion proteins as illustrated. (*b*) Chelator lipid bilayer tethered by histidine-terminated alkylthiols. (*c*) Chelator lipid bilayer tethered by histidine-functionalized lipid monolayer.

tor-mediated docking resembles many interesting features of vesicle docking, virus fusion, or cell adhesion.

MATERIALS AND METHODS

Materials

Pam_3Cys-$(Gly\text{-}Ser)_8$-His_6-OH (Pam_3Cys-OH: *N*-palmitoyl-S-(2, 3-bis(palmitoyloxy)-(2R, S)-propyl)-(R)-Cys-OH), MHDA-TEG-OH (MHDA-OH: 16-mercaptohexadecanoic acid; TEG-OH: α-(β-aminopropionyl)-ω-(carboxymethyl-carbamoyl)-tetraoxyethylene), and MHDA-TEG-His_6-OH were prepared by solid phase chemistry. The histidine-tagged polypeptide H-$(Gly\text{-}Ser)_{10}$-His_6-OH (molecular weight = 2822 g/mol) was synthesized by fluorenylmethoxycarbonyl (Fmoc) solid-phase chemistry using standard protocols. *N*-palmitoyl-S-(2, 3-bis(palmitoyloxy)-(2R, S)-propyl)-(R)-Cys-OH) (Wiesmüller et al., 1983) and trityl-MHDA-OH were coupled manually after carbodiimide activation. All products were characterized by reversed-phase high-performance liquid chromatography and electro-spray mass spectrometry. The chelator lipid N_α,N_α-bis[carboxymethyl]-*N*-[(dioctadecylamino)-succinyl]-L-lysine, NTA-DODA, and Fmoc-TEG-OH were synthesized as described (Schmitt et al., 1994; Mack et al., 1999). The structures of the compounds used in this study are summarized in Fig. 2. 1-Stearoyl-2-oleyl-*sn*-glycero-3-phosphatidylcholine (SOPC) was purchased from Avanti Polar Lipids (Birmingham, AL). All other solvents and chemicals were ordered from Fluka (Neu-Ulm, Germany) in pro-analysis quality. If not stated otherwise, experiments were carried out in sterile-filtered and degassed HEPES-buffered saline (HBS: 10 mM HEPES, 150 mM NaCl, pH 7.5). In some experiments, the chelator lipid was preloaded with nickel ions (Ni-NTA-DODA) in chloroform/methanol (6:1, v/v) by adding equimolar amounts of nickel chloride dissolved in methanol. Vesicles were prepared by mixing appropriate molar ratios of lipids in chloroform/methanol, evaporation of the solvent in vacuum, swelling in HBS (0.2 mM total lipids), and repeated extrusion through 100-nm filters (LiposoFast-Basic, Avestin, Ottawa, Canada).

Membrane formation

Supported membranes were prepared by vesicle fusion (0.2 mM lipid in HBS) onto a self-assembled layer of octadecylthiol on gold. Before vesicle addition, the surface was rinsed several times with 40 mM octyl-β-D-glucopyranoside at a flow rate of 10 μl/min. The process of lipid adsorption was followed by SPR spectroscopy at a flow rate of 2 μl/min using a BIAcore-X instrument (BIAcore AB, Uppsala, Sweden). If not otherwise stated, buffer exchange and binding of histidine-tagged biomolecules were performed at a flow rate of 10 μl/min. All experiments were carried out at 22°C. The change of the SPR signal is given in resonance units, which are related to the shift in refractive index or in layer thickness of the interface. According to the BIAtechnology handbook (BIAcore AB) 1000 resonance units (RUs) are equivalent to 1 ng protein/mm^2.

Atomic force microscopy

AFM measurements were performed on gold-coated glass slides (Pioneer Chip, BIAcore AB), which were functionalized by thiols. These thin films contain 90 mol % MHDA-TEG-OH (matrix thiol) and 10 mol % MHDA-TEG-$(His)_6$-OH (histidine-tagged thiol). Self-assembly of thiols (1 mM) was performed from solutions of ethanol/H_2O (9:1, v/v) overnight. Slides were extensively washed with ethanol and water to remove the excess of thiols. These SAMs were incubated for 2 h in 0.2 mM vesicle solution (90 mol % SOPC and 10 mol % chelator lipid). Chips were mounted into a Nanoscope III AFM (Digital Instruments, Santa Barbara, CA). Lipid layers

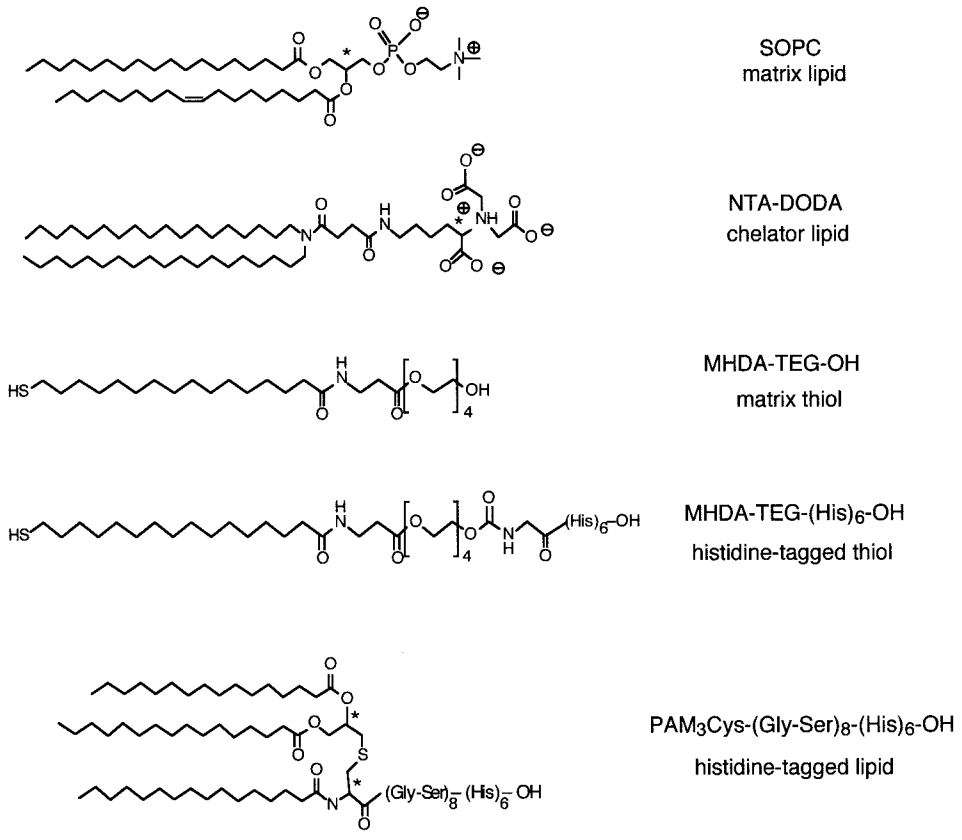

FIGURE 2 Chemical structures of functionalized lipids and thiols used in this study.

were washed with water to remove nonspecifically bound vesicles by osmotic stress. Finally, water was exchanged by HBS to image the lipid layers under the same conditions as used for the SPR measurement. Cantilevers were oxide-sharpened silicon nitride tips (Digital Instruments) with a spring constant of 0.58, 0.32, 0.12, and 0.06 N/m, respectively. For scratching, stiff tips were used. Imaging was performed in contact mode with the softest tips at a scan rate of 3 Hz. In addition, tapping mode was employed to reduce distortion of soft samples. However, no significant differences were observed between both modes.

RESULTS

Formation of planar hybrid membranes

For comparison with more complex membrane architectures, we generated lipid monolayers on hydrophobic SAMs of alkylthiols. These planar hybrid membranes are formed by fusion of SOPC vesicles containing metal-chelating lipids. The vesicle solution (0.2 mM SOPC containing 10 mol % Ni-NTA-DODA) was added three times (Fig. 3a). Almost no increase of the SPR signal was observed after the second (8% increase) and third (2% increase) incubation. The hybrid bilayers were incubated in buffer for an additional 2 h to allow equilibration. The interface was rinsed with deionized water to remove adsorbed vesicles by osmotic stress. Vesicle fusion leads to an increase of 2800 ± 300 RUs. This value is in very good agreement with data previously published, demonstrating that a lipid monolayer is formed (Dorn et al., 1998b, 1999). These planar hybrid membranes will serve as a reference for the planar lipid bilayer tethered by ligand-receptor pairs as described below.

Formation of lipid bilayers tethered via histidine-tagged thiols

Metal-chelating lipids have been found to be very useful for the specific immobilization, orientation, and two-dimensional crystallization of histidine-tagged proteins at lipid interfaces. Here, we have extended this concept for the specific tethering of biomembranes. In the first step, we have developed functionalized interfaces by self-assembly. These films are tailored by (6xHis) tags to capture chelator-lipid-modified membranes by metal-affinity binding. We used two different approaches to create (6xHis)-tagged interfaces with well defined compositions. One is based on SAMs composed of histidine-tagged thiols and PEG-thiols (1:9, mol/mol; Fig. 2). Similar PEG-thiols have been used to block nonspecific protein adsorption (Prime and Whitesides, 1991). It has been further demonstrated that the composition of the omega-functionalized long-chain alkylthiols in solution and in SAMs is identical (Tengvall et al., 1998).

These (6xHis)-functionalized SAMs were incubated with SOPC vesicles. To allow specific docking and fusion, vesicles were doped with Ni-NTA-DODA (10 mol %). Using SPR, the lipid adsorption was followed in real-time. The structure of the lipid layer was analyzed by AFM (see below). During incubation with vesicle solution (0.2 mM lipid), the SPR signal increased by 4200 ± 400 RUs (Fig. 3 *b*). Almost no further increase was observed during the second (5% increase) and third (2% increase) incubation periods. After removal of weakly adsorbed lipid, a stable resonance signal of 4200 ± 400 RUs was achieved. The kinetics of lipid adsorption at histidine-tagged SAMs is very similar to the formation of a planar lipid monolayer at hydrophobic alkylthiols (Fig. 3 *a*). In both cases, layer formation is completed within 30–60 min. Weakly bound lipid structures were removed by osmotic swelling. This is in good agreement with studies from other groups (Cooper et al., 1998). However, the resonance signal is almost twice as much as that of the hydrophobic SAMs, indicating that bilayers on histidine-tagged SAMs are formed.

Structure of lipid bilayers tethered via histidine-tagged thiols

The (6xHis)-functionalized SAMs were characterized by AFM. This technique is an excellent tool to image solid as

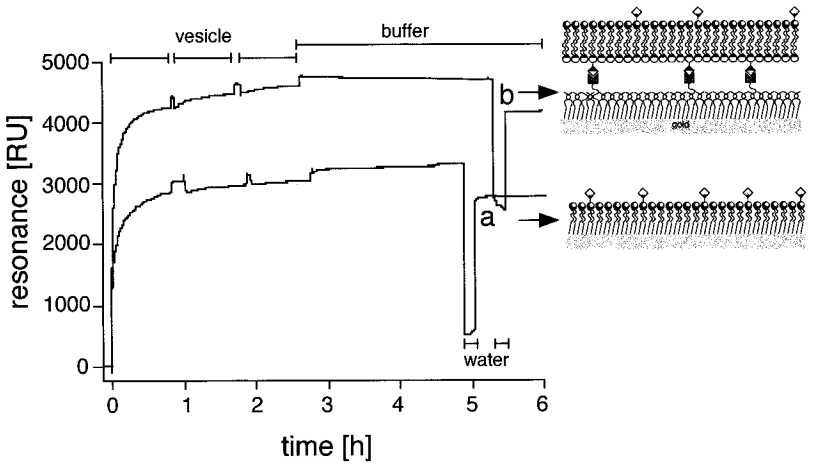

FIGURE 3 The formation of lipid layers on SAMs. Lipid adsorption from vesicular solution (0.2 mM lipid) was followed by SPR spectroscopy. The lipid vesicles containing 90 mol % SOPC and 10 mol % Ni-NTA-DODA were added three times. After 2 h of equilibration in HBS buffer, weakly adsorbed lipid structures were removed by washing with deionized water. The lower curve (*a*) shows the formation of a lipid monolayer on a hydrophobic self-assembled alkylthiol monolayer. Lipid adsorption to histidine-terminated SAMs is shown by the upper curve (*b*).

well as soft organic interfaces ranging from thin films to living cells (Radmacher et al., 1992). As a first reference, the bare gold substrate (Pioneer Chip, BIAcore AB) was imaged. Characteristic islands known for gold evaporated on flat surfaces were observed (data not shown). The root mean square (RMS) deviation of the roughness scanned over 100 μm^2 is below 0.5 nm. This observation is in good agreement with data previously reported (Liu and Brown, 1997). SAMs containing PEG- and (6xHis)-thiols (9:1) were imaged in HBS buffer employing the same conditions as in the SPR experiment. Homogeneously flat layers were obtained (Fig. 4 *a*). It should be emphasized that even over large areas (100 μm^2) no defects on the surface were observed. The RMS deviation of the surface roughness scanned over 100 μm^2 is below 0.4 nm. Owing to the polymeric headgroup, the SAM smears out the surface roughness of the bare gold surface. The thickness of the adsorbed thiol layer was analyzed by scratching experiments. Using a stiff AFM tip, the thiol film was removed at small areas. Line scans across several defects reveal that the depth was approximately 2.5 $\pm$ 0.5 nm. This corresponds well with the estimated thickness of the thiol monolayer.

For AFM analysis of the tethered lipid layers, the (6xHis)-functionalized SAMs were incubated for 2 h with vesicle solution (0.2 mM SOPC doped with 10 mol % Ni-NTA-DODA). The conditions were kept identical to those of the SPR experiment. The surface was intensively rinsed with deionized water to remove weakly adsorbed structures. After this harsh washing procedure, typically lipid patches of >16 μm^2 were imaged in HBS buffer as shown in Fig. 4 *b*. The lipid patches were found to be homogeneous in height (7 $\pm$ 1 nm). This value is in very good agreement with the thickness of a lipid bilayer supported by an ultrathin water layer (Sackmann, 1996). By scratching experiments, the overall height of the composite layers including the functionalized SAM was determined to be 9.5 $\pm$ 1 nm (Fig. 4 *b*). Outside the lipid layers the thickness of the functionalized SAM was determined to be 2.5 $\pm$ 0.5 nm, demonstrating that the surface topology of the SAM has not been changed during the procedure of vesicle fusion and washing. Lipid patches observed by AFM correspond to more than 500 fused vesicles, assuming a hard sphere surface with a diameter of 100 nm. On the basis of these results we conclude that productive vesicle fusion and formation of a continuous planar lipid bilayer occurred.

Formation of lipid bilayers tethered via histidine-tagged lipids

Applying an alternative approach, surface functionalization was achieved simply by fusion of SOPC vesicles doped with histidine-tagged lipids (Fig. 2). Similar to the histidine-terminated thiols, these functionalized lipids serve as spe-

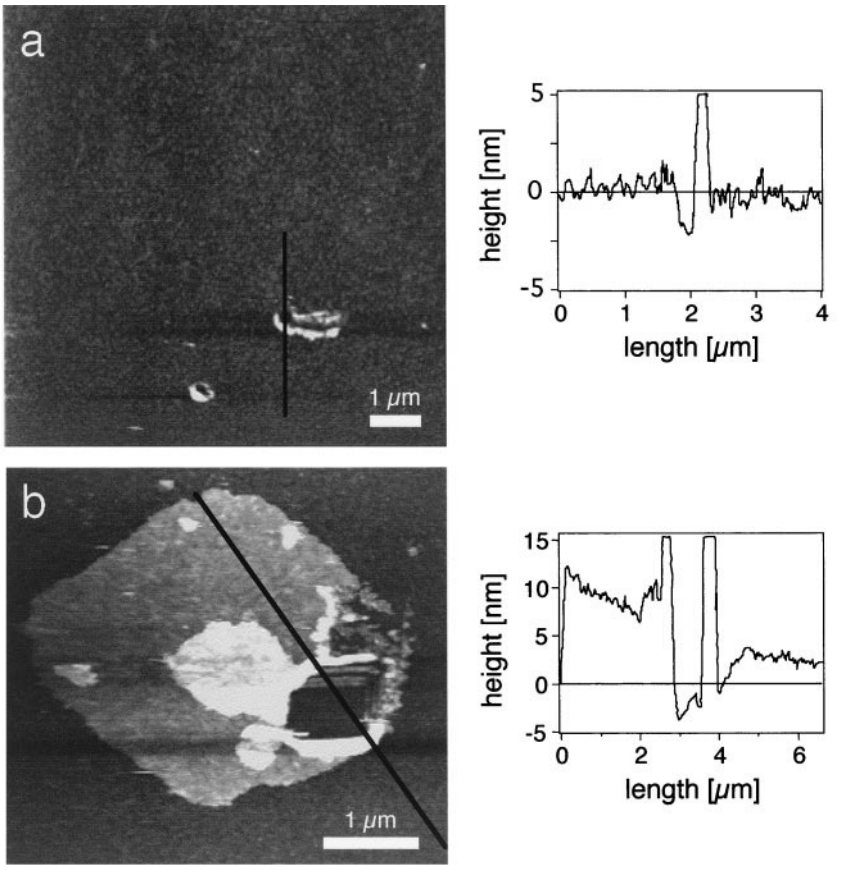

FIGURE 4 (*a*) AFM image of a self-assembled monolayer containing 90 mol % PEG-thiol and 10 mol % histidine-tagged thiol. By scratching on the surface with the AFM tip defects were produced. The removed material was deposited close to the defect. For details see Materials and Methods. A section analysis was taken along the black line. The defect has a depth of 2.5 $\pm$ 0.5 nm. (*b*) AFM images of the same histidine-terminated SAM after docking and fusion of SOPC vesicles containing 10 mol % Ni-NTA-DODA. Scratching with the AFM tip produced a defect of 1 $\mu m \times 1$ μm. A section analysis was taken along the black line. The scratching area has a depth of 9.5 $\pm$ 1 nm. Scratching experiments at regions outside of lipid patches demonstrated similar results as shown in *a*.

cific interaction partners for metal-chelating lipids. Note that both approaches lead to similar histidine-functionalized interfaces. However, both histidine-tagged interfaces differ in the lateral mobility of their compounds. Alkylthiols are more or less fixed, whereas lipids diffuse laterally within the monolayer. Formation of the histidine-functionalized lipid monolayer was followed by SPR. Addition of SOPC vesicles containing 10 mol % histidine-tagged lipids leads to the formation of hybrid membranes as described before. The (6xHis)-functionalized monolayer was allowed to equilibrate for 2 h.

In the next step, lipid vesicles (0.2 mM) were incubated with this histidine-functionalized lipid interface following the same procedure as described for histidine-tagged thiol monolayers. Incubation of vesicles containing 10 mol % of the nickel-loaded chelator lipid Ni-NTA-DODA resulted in a shift in the resonance signal of 4600 ± 400 RUs (Fig. 5 *a*). Within the range of error this is identical to the value obtained for the lipid membrane tethered by the histidine-tagged SAMs, indicating that a lipid bilayer is formed on the histidine-functionalized interface. Formation of these histidine-tethered membranes is highly specific and requires the presence of the histidine tags at the target lipid interface and metal-loaded chelating lipids at the vesicular lipid interface. When SOPC vesicles containing chelator lipids were used in the absence of nickel ions (50 μM EDTA), no lipid adsorption was observed (Fig. 5 *b*). In addition, pure SOPC vesicles do not interact with the histidine-functionalized interface (Fig. 5 *c*). Most strikingly, nonspecific adsorption is below 1% in all controls, demonstrating that vesicle docking and fusion can be switched on and off by complex formation of the chelator lipid.

Specific docking and surface accessibility to the tethered bilayer

Specific binding of histidine-tagged proteins is a simple and most practical feature to examine the function and geometry of the lipid interface. As a reference we used the well characterized planar lipid monolayers on alkylthiols (see Fig. 1 *a*). The properties of these chelator lipid membranes (10 mol % Ni-NTA-DODA) were probed by specific binding of a polypeptide containing a histidine tag at its carboxy terminus (H-$(Gly\text{-}Ser)_{10}$-His_6-OH). To prevent trapping of divalent ions from the buffer, the chelator lipid monolayer was incubated with 100 mM EDTA (pH 7.5) and immediately loaded with nickel chloride before a binding experiment was performed. An increase of the SPR signal was observed after addition of the histidine-tagged polypeptide (Fig. 6 *a*). After 6 min, equilibrium and saturation of all binding sites is reached at 300 ± 20 RUs, corresponding to a surface concentration of 0.3 ng/mm^2 (~0.1 pmol/mm^2). The immobilization was stable over time, as reflected by the slow dissociation kinetics. Typically, interfaces containing 10 mol % metal-chelating lipids show stable immobilization of histidine-tagged proteins. The binding and dissociation process cannot be fitted by monoexponential kinetics, indicating that (6xHis) proteins/peptides are immobilized due to multivalent trapping (Dorn et al., 1998b, 1999). Desorption was achieved by addition of imidazol or EDTA (100 mM each). Thereby, the surface was completely regenerated (nonspecific binding is below 3% in all experiments) and a new binding experiment could be performed.

The surface area and geometry of the tethered lipid layer should affect the binding process of the (6xHis) fusion proteins. Hence, we compared the different tethered membranes (Fig. 1, *b* and *c*). The composition of lipid layer as well as the treatment of the interface was identical as described for the planar hybrid monolayer. Binding and dissociation process appears to be similar to the planar hybrid monolayer, which serves as a reference. Binding of the histidine-tagged peptide reached equilibrium at 230 ± 15 RUs within 6 min (Fig. 6 *b*). The histidine-tagged peptide could be removed from the surface by competition with imidazol (data not shown). Finally, the membranes, which are tethered via (6xHis)-functionalized lipids, were ana-

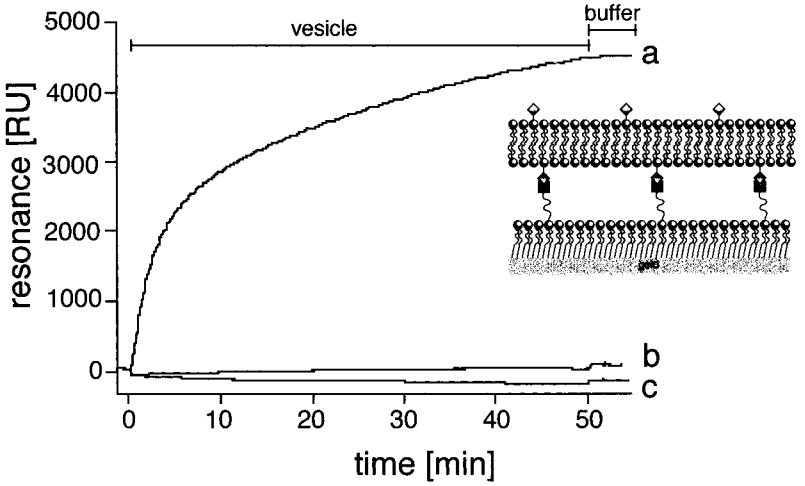

FIGURE 5 (*a*) Formation of lipid layers on a histidine-functionalized interface followed by SPR spectroscopy. SOPC vesicles containing 10 mol % nickel-loaded chelator lipids were incubated with histidine-functionalized surface. After docking and fusion the surface was equilibrated in HBS buffer. Vesicles (0.2 mM lipid) were added in HBS. (*b*) Under identical conditions, the same vesicle preparation was added in the presence of EDTA. (*c*) Lipid adsorption of SOPC vesicles containing no chelator lipid. Histidine-functionalized interfaces were generated by fusion of SOPC vesicles containing 10 mol % of the histidine-tagged lipid onto a hydrophobic SAM of alkylthiols. All measurements were performed in HBS at pH 7.5 except the fusion of SOPC vesicles in HBS at pH 8.0.

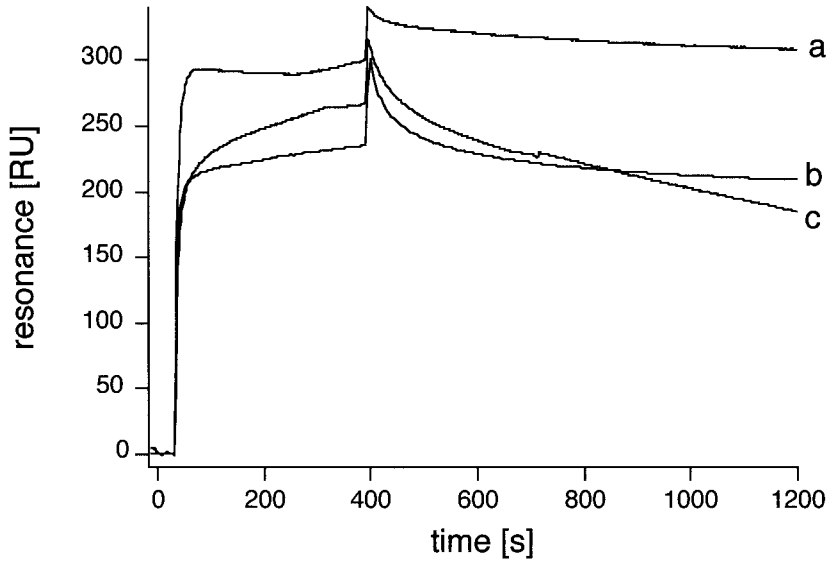

FIGURE 6 Comparison of the immobilization of a histidine-tagged peptide at chelator lipid interfaces of different architectures as illustrated in Fig. 1: (*a*) planar lipid monolayer on hydrophobic SAMs of alkylthiols, (*b*) tethered membranes on histidine-tagged SAMs, (*c*) or histidine-tagged lipid monolayers. After loading the chelator lipid interface with nickel ions, binding and dissociation kinetics of the histidine-tagged polypeptide (20 μM) were analyzed by SPR spectroscopy. Addition of histidine-tagged peptide and the washing procedure with HBS buffer are indicated.

lyzed (Fig. 6 *c*). The change of the SPR signal was similar to the binding and dissociation kinetics observed before. Equilibrium of binding is reached at 270 $\pm$ 20 RUs. Once again immobilization was stable over time. Dissociation was achieved by injection of imidazol or EDTA (data not shown). The immobilization of the histidine-tagged peptide at the metal-chelating lipid layer is highly specific, because no binding was observed in the presence of EDTA. Based on these results we conclude that these solid-supported lipid layers show very similar surface geometry and docking sites.

DISCUSSION

Supported lipid layers open the possibility of studying the structure and function of biomembranes by surface-sensitive techniques including also single-molecule analysis. Chelator lipids have proven to be a very useful approach for the specific immobilization, designed orientation, and two-dimensional crystallization of histidine-tagged proteins (Tampé et al., 1996). In this study, we pioneered the NTA-histidine-tag system for the specific tethering of lipid membranes. In comparison with planar lipid monolayers (Fig. 1 *a*), MAT membranes lead to comparable binding kinetics of (6xHis) fusion proteins. Thus lipid interfaces with similar docking sites and geometry are generated. It is no longer possible to fit association and dissociation kinetics by monoexponential functions and to quantify adsorption in terms of a simple intrinsic dissociation constant if multivalent interactions occur (Dorn et al., 1998b). The binding values differ slightly, which can be explained by two reasons. First, the evanescent field decays exponentially at the sensor surface. Thus, binding of histidine-tagged peptides will result in a smaller signal for tethered membranes in comparison with planar monolayers. Second, in contrast to planar lipid monolayers, the composition of the tethered membrane with respect to the proximal and distal lipid layer may be different before and after vesicle fusion. It is quite reasonable that the formation of ligand-receptor pairs deplete metal-chelating sites in the distal layer. Such separations are described in literature but still poorly understood (Andelman, 1995). Immobilized histidine fusion proteins can be removed from the tethered membrane by adding EDTA (nonspecific binding is below 3%). This is consistent with our observations that no binding of histidine-tagged polypeptides occurs when binding experiments are performed in the presence of EDTA. Taking these considerations into account we conclude that the binding process of histidine-tagged proteins to these tethered membranes and planar monolayer are identical.

In contrast to histidine-tagged proteins, tethered membranes are remarkably stable toward treatment with EDTA or imidazol. This is not surprising for EDTA, which is membrane impermeable. On the other hand, imidazol is expected to be partially membrane permeable. In principle, however, very high concentrations of the monovalent compound are needed to compete for the histidine tags, which are presented at high surface concentrations. Most interestingly, the binding constant for the NTA/histidine-tag system is moderately weak. It was determined to be in the micromolar range (Nieba et al., 1997; Dorn et al., 1998a). One can speculate that the observed stable immobilization is due to multivalent interactions of the tethered lipid bilayers with their supports. The cooperative binding arises from the proximity of ligands on the support. The ligand and receptor densities together with geometric considerations can be applied to determine the maximum number of interactions leading finally to an apparent reduction in the observed dissociation constant. Initial stages of docking and fusion where contact is engineered between vesicle and support

will be dominated by mechanic and viscoelastic properties of the lipid vesicles rather than molecular kinetics. With regard to the driving forces of fusion, which are not dominated by a high surface energy of the interface, many pinning events force the vesicle to fuse (Bunjes et al., 1997). The influence of the surface density of receptor-ligand pairs is of great interest in our ongoing research. However, it is known that reaching a point below cooperative effects in recognition processes at membranes is difficult to achieve (Horan et al., 1999). When the fluidity of the chelator lipid vesicles was decreased, vesicle adsorption but no fusion was observed, consistent with the observation that formation of supported bilayers is inhibited if lipids are in their crystalline state (Rädler and Sackmann, 1993; Sackmann, 1996).

Once multiple bonds have formed, the theory suggests that the intrinsic dissociation constant has little influence on the stability of attachment. Given that each interaction can be assigned a bond strength, the speed at which these bonds can form and break become important (Hubble, 1997; Mammen et al., 1998). Such behavior resembles many features of a cellular membrane, such as vesicle fusion, cell adhesion, and docking of viruses. The stable immobilization appears to be an indirect proof that the tethered lipid bilayer lack defects on the mesoscale. Otherwise the high concentrations of EDTA or imidazol may disrupt the ligand-receptor interactions. Lowering the pH to release the ligand-receptor pairs will have many additional effects on the interface that are difficult to separate. In general, desorption of supported membranes is hard to control if multivalent interaction sites have to be broken.

In the AFM experiments we were able to image tethered lipid bilayers on histidine-functionalized SAMs. So far, only a few AFM studies have been carried out on solid-supported lipid bilayers (Jenkins et al., 1999; Dorn et al., 1999). The overall thickness of the composite system was determined to 9.5 $\pm$ 1 nm. These data are in accordance to thickness of lipid bilayers (5 nm) (Winger and Chaikof, 1998) separated by an ultrathin water film (1–2 nm) (Sackmann, 1996). The thickness of a supported bilayer (5–7 nm) has been characterized by various methods including AFM, reflection interference contrast microscopy, and neutron- and x-ray diffraction techniques (King and White, 1986; Rädler and Sackmann, 1993; Hui et al., 1995; Sun et al., 1996). By scanning over larger areas ($>$1000 μm^2), we observed that 40% of the surface was covered with lipid patches even after very harsh external washing procedures. Unfortunately, the lipid interfaces cannot be analyzed by AFM directly after the SPR experiment where buffer exchange and the washing procedure are optimized. Due to these limitations, layers tethered via histidine-tagged lipids cannot be analyzed by AFM. Presently we are working on an efficient method to transfer the lipid interface under buffer from the SPR to the AFM set-up.

We are very much concerned about the question of whether planar lipid bilayers or vesicle layers are formed. Several lines of evidence support the conclusion that planar supported membranes are formed. First, adsorption values are highly reproducible and correlate with the surface coverage and thickness of lipid mono- and bilayers. The formation of a vesicle layer results in higher surface coverage and resonance signals. Second, the formation of these tethered membranes as well as their subsequent binding behavior of (6xHis) fusion proteins are highly specific. This is in contrast to the situation in which layers of lipid vesicles are formed. Third, the thickness of the tethered membrane analyzed by AFM corresponds to a lipid bilayer. All experiments were performed on self-assembled alkylthiol-gold surfaces, which are nearly defect-free and easy to modify. However, these tethered bilayers cannot be simply studied by fluorescence microscopy applying fluorescence recovery after photobleaching (FRAP) techniques as additional proof for bilayer formation.

In this study, a novel approach for tethering of lipid membranes was introduced. The key-lock principle between histidine-tag and metal-complex lipids was successfully adapted for docking and tethering of lipid membranes. Vesicle docking and fusion is highly specific as pure SOPC vesicles or vesicles containing unloaded chelator lipids do not significantly interact with the functionalized support. The generic approach is highly flexible and can be in principle adapted to liposomes containing membrane proteins such as histidine-tagged receptors, transporters, or channels. One can envision that these tethered lipid layers are most practical to study the recognition, transport, and signaling processes at biomembranes.

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